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Development of a Quantitative TaqMan™-PCR Assay and Feasibility of Atmospheric Collection for *Coccidioides immitis* for Ecological Studies

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Lawrence Livermore National Laboratory

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Development of a Quantitative TaqMan™-PCR Assay and Feasibility of Atmospheric Collection for *Coccidioides immitis* for Ecological Studies

Final Report on LDRD Project 01-ERD-090

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February 01, 2002

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TABLE OF CONTENTS

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Development of a Quantitative TaqMan™-PCR Assay and Feasibility of Atmospheric Collection for *Coccidioides immitis* for Ecological Studies

Introduction

Understanding and then modeling the complex relationships between environmental, climatological, and biological systems can present great opportunities for developing effective strategies to prevent outbreaks of environmentally linked infectious disease. For example, a report from the American Academy of Microbiology (Rose et al., 2001) points out that including climatic signals and ecological triggers into the decision making process for disease management, is going to be essential for developing a proactive approach for protecting public health, and circumventing the practice of having to wait for clinical cases to appear before any action is taken. To meet this grand challenge, specific and sensitive analytical techniques and effective collection devices are required for finding the microorganisms of concern in environmentally relevant media (air, soil, water, vegetation, etc.), and a multidisciplinary approach is needed for evaluating all of the environmental and biological data and building the applicable predictive models.

In this study, the unique capabilities in biotechnology and environmental science available at Lawrence Livermore National Laboratory (LLNL) are combined with the distinguished and highly regarded expertise for clinically investigating and treating infectious disease at the Department of Microbiology and Immunology in the School of Medicine of the University of California, Davis, in order to develop, standardize, validate, and test safely the feasibility of applying advanced polymerase chain reaction (PCR) technology and new air-sample collection media that would be appropriate for addressing comprehensively the environmentally linked, medically important infectious disease Valley Fever (coccidioidomycosis). The responsible agent for this disease is the airborne spore (arthroconidia) of the pathogenic fungus *Coccidioides immitis*, which is a microorganism that is endemic to California, Arizona, and the southwestern United States, and also is identified as a select (biological) agent in the federal *Anti-Terrorist and Effective Death Penalty Act*. Successful demonstration of these tools in this study will place this multidisciplinary team in a credible position to proceed with additional research designed to determine the climatic signals and ecological triggers that would be associated with the presence of this microorganism environmentally and that would correlate with subsequent outbreaks of Valley Fever clinically. Results from such future research would then provide the information needed for environmental intervention of the disease occurrence, well before clinical cases appear. The technology and modeling developed for such a study also could be used for determining the ecology of other environmentally linked, medically important infectious diseases that occur naturally or that might be introduced deliberately into environmental media indoors or outside.

The following approach was taken to achieve the technological objectives of this study. First, the protocols for the TaqMan™-PCR assay were enhanced to achieve the superior specificity and sensitivity required for quantifying, from a DNA signature, those

C. immitis spores that are present in calibration samples (consisting of known quantities of pure culture inoculated onto air-filter concentrate, and then removed, and the DNA extracted) and those that are present within the calibration range on air filters obtained from the field and handled similarly. Second, the feasibility of using advanced nuclepore air-filter media to collect the spores from ambient air in *C immitis* endemic areas in the Central Valley of California was evaluated. These membranes permit the physics of high-volume air sampling to be used to filter larger amounts of air than previously possible for detecting airborne microorganisms. Thus, the higher volume of air flow improves the likelihood of capturing on the filter any *C. immitis* spores resuspended from nearby soil, where this microorganism grows prior to sporulation, and where its exact location can be elusive under any environmental conditions. Finally, experiments were conducted in the Department of Microbiology and Immunology at the School of Medicine of the University of California, Davis (UCD), to demonstrate that *C. immitis* spores can be killed, whether in pure culture or on air-filter media, to ensure safe handling during laboratory processing and analyses.

Materials and Methods

The methods developed for this study apply to its three different objectives: 1) enhancement of the TaqMan™-PCR assay for quantitative detection of *C. immitis* spores, 2) evaluation of high-volume air sampling using a nuclepore air-filter membrane, and 3) demonstration of spore killing.

TaqMan™-PCR Assay for Calibration and Detection of C. immitis

The inter-genic spacer region of the *C. immitis* DNA was targeted for specific amplification (Greene, 2000) by a quantitative polymerase chain reaction (PCR) assay made possible by TaqMan™ technology (Perkin-Elmer Applied Biosystems Division, 1996). All amplification reactions were performed on an ABI 7700 Sequence Detection System using 0.2 µM primers Its-C1A (5′ CAT CAT AGC AAA AAT CAA AC 3′) and Its-C2 (5′ AGG CCC GTC CAC ACA AG 3′) with 1.25U Platinum Taq polymerase (Invitrogen, Carlsbad, CA), 6 mM $MgCl_2$, 0.8 mM dATP, dCTP, and DGTP with 1 mM dUTP, 400 nM cocci1 Fam-Tamra probe, and 5 µL of extracted DNA, all in Platinum Taq PCR buffer. Optimized PCR conditions were 1 cycle of 1 min at 95° C, and 35°cycles of 15 sec at 95° C followed by 1 min at 63° C.

To obtain DNA from the spores of *C. immitis* collected on air samples, the spores must be removed from the air-filter membrane and the DNA extracted. The following procedure was employed to remove any *C. immitis* spores from the air-filter membrane.

1. Air filters are cut in half using sterile scissors and tweezers: one half is processed and the other is archived at –20° C.

2. The half filter that undergoes processing is then cut into 20 to 30 small strips using sterile scissors and tweezers and placed in a 50-mL conical Falcon tube containing 45 mL of phosphate buffered Tween solution (PBT solution is 0.003% Tween-20, 17 mM KH_2PO_4, and 72 mM K_2HPO_4), vortexed horizontally for 1.5 minutes at maximum power [VWR, Well block combi-shaker (VWR, West Chester, PA)] and sonicated on power level 9 [VWR, Aquasonic Model 75D (VWR, West Chester, PA)] at room temperature for 10 min.
3. After sonication, the tube is vortexed for 5 seconds and the liquid is poured into a clean Falcon tube. To remove any particles remaining on the filter pieces in the original Falcon tube, the wash is repeated by adding an additional 45 mL of PBT and processed as in step 2. That tube is again vortexed for 5 seconds and the liquid poured off into a second clean Falcon tube.
4. Both sample washes are then centrifuged for 30 minutes at 4° C using a Jouan CR422 centrifuge (swinging bucket, tabletop centrifuge) set at a speed of 3500 × g (4000 rpm). The supernatants are then discarded and each pellet is transferred to a 1.5-mL microcentrifuge tube.
5. The 1.5 ml tubes are centrifuged for 8 min at a speed of 16,000 × g. Supernatants are poured off and the resulting pellets are combined with any remaining supernatant for a total volume of 200 µl. This represents the air sludge or air-filter concentrate that will undergo a procedure for extracting the DNA from any C. immitis or other microorganisms that may have been collected and be present.

The method of Miller et al. (1999) was modified to extract the DNA from any *C. immitis* spores that may be present. This method involves the following steps.

1. A combination of 300 µL of Miller Phosphate Buffer, 300 µL of Miller SDS, 300 µL of Phenol Chloroform Isoamyl alcohol, and 100 µL of the air sludge are added to a 2 mL tube containing 0.9 g of silica-bead mix.
2. Lysis of microorganisms in the air sludge is accomplished by placing the tube in a Bio101 Fast Prep 120 machine for two bursts of 45 seconds at 6.5 speed.
3. The tube is then centrifuged at 16,000 × g for 5 minutes.
4. The aqueous phase is recovered and poured into a new 1.5 mL tube (volume is about 700 µL).
5. To this liquid is added 700 µL of chloroform, and the tube is then vortexed for 10 sec, and subsequently centrifuged at 16,000 × g for 3 minutes.
6. The supernatant is removed to a 2-mL tube without disturbing the chloroform layer underneath (volume is approximately 550 µL).
7. Two equivalent volumes (about 1100 µL, if 550 µL of aqueous supernatant was collected) of MoBio-solution S3 are then added to the tube and the contents are vortexed for 5 sec.

8. The MoBio Spin Filter is added with a 700 µL aliquot and centrifuged at a speed of 10,000 × g for 30 seconds. This step is repeated until all supernatant has been spun through the filter.
9. The filter is then washed with 300 µL of MoBio Solution S4 and centrifuged at a speed of 10,000 × g for 30 seconds.
10. The supernatant is then discarded.
11. The sediment is centrifuged again at 10,000 × g for 1 min.
12. The spin filter is then placed into a clean 1.5 mL tube—splashing of Solution S4 onto the spin filter is avoided.
13. Next, 75 µL of MoBio Solution S5 are added to the center of the white filter membrane.
14. This mixture is then centrifuged for 30 seconds at a speed of 10,000 × g to elute DNA.
15. Next, the spin filter is discarded and the DNA is what remains in the tube.
16. The preparation of a S-200 Sephacryl column is performed by first vortexing the column to resuspend resin, snapping the bottom off, loosening the cap, turning it, and placing the column in a 2 mL catch tube. The column is then spun for 1 minute at 3000 rpm (735 × g) and subsequently placed into a new 2 mL catch tube.
17. The DNA sample from step 15 is then applied to the column, and the sample is collected in the 2 mL tube underneath. The DNA is stored at –20° C until it is applied (it will degrade if stored at 4° C).
18. A sample that is too dilute can be dried down with a speed vac for 15 minutes to reduce the final volume.

High-Volume Air Sampling Using a Nuclepore Air-Filter Membrane

The air sampler (Figure 1) selected for evaluation in this study is a modified high-volume dust-particle collector, that is operated by a 3-stage brushless motor blower that is capable of a maximum unimpeded flow rate of 100 m^3/hr (58 cfm). The sampler is powered by either electricity or a gasoline powered generator.

Air particulates are collected on a nuclepore track-etched (1-µm pore size), 8"×"10-in (about 20 × 25-cm), polyester-membrane filter (Osmonics, Westborough, MA). The spores (arthroconidia) of *C. immitis* are typically 2- to 5-µm, cylindrical or barrel shaped objects (Pappagianis, 1988). Therefore, if such spores are present in the collected air, they will be deposited on the surface of the 1-µm pore-size filter.

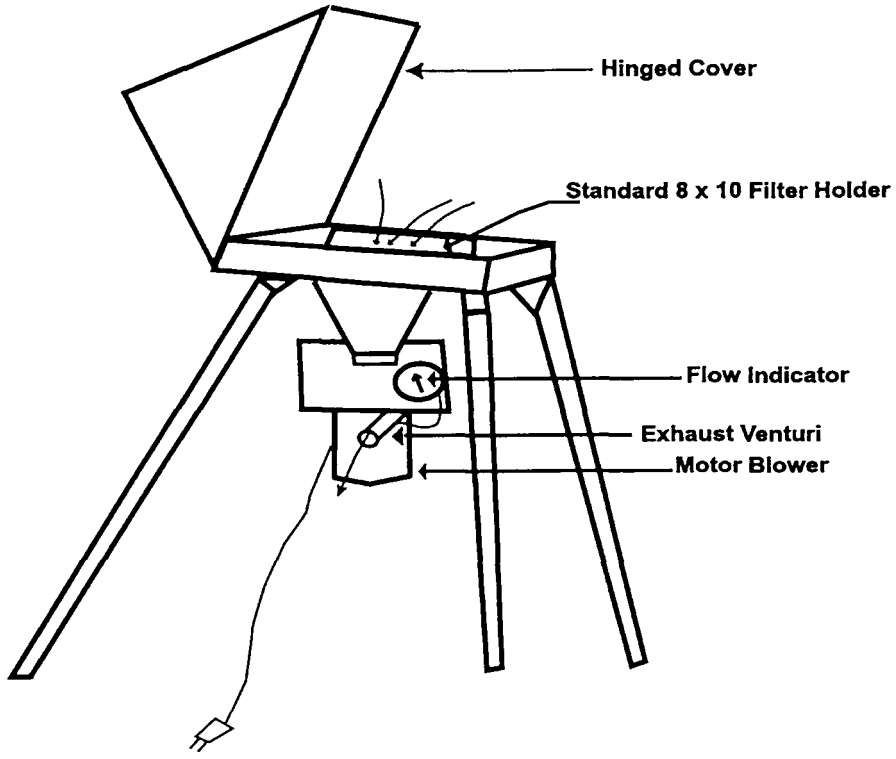

Figure 1. Diagram of air-filter apparatus evaluated for collecting *C. immitis* spores on nuclepore membrane air-filters at two locations in Bakersfield, CA, in August 2001.

The system is operated for 24 h at an average flow rate of 60 m^3/hr. Thus, an average of 1.4×10^6 L of air pass over a filter in 24 h, and then the filter is removed. After removal, the air filter is cut in half and both halves are stored at –20° C. One half is then steam killed for subsequent TaqMan™-PCR assay analysis, while the other half is archived.

The half-filter selected for analysis is then washed to recover all collected material. That air-sludge then undergoes treatment for extraction of genomic deoxyribonucleic acid (DNA) from any *C. immitis* spores that may be present. The amount of DNA is then related to the number of microorganisms present in the collected air.

During the summer of 2001, four air samplers and the air-filter media just described were deployed at two different sites in Bakersfield, CA (Kern County) in order to collect 24-h air samples in a region considered endemic for *C. immitis*. One site was minimally disturbed cattle grazing ranch land in the vicinity of a geological landmark known as Sharks Tooth Hill. The other site was agricultural land that was cultivated. The agricultural land was located about 13 miles (approximately 22 km) to the west of the ranch land. On the ranch land, the four samplers were staggered over a rectangular area of about 150 m by 100 m that included a flat plateau on one end and a gentle slope on the other, with the separation between the two samplers that were farthest apart equal to the 150-m distance. The agricultural land was a rectangular field that was 400 m by 800 m in

dimensions. The four samplers were deployed toward the central part of the field roughly in a line, with the furthest separation between the two most distant samplers equal to 60 m. For logistical reasons, the four samplers on the ranch land were able to be operated for two 24-h periods ending on August 28 and August 29, 2001, but the four samplers on the cultivated agricultural land could only be operated for one 24-h period ending on August 29, 2001. Thus, two sets of four air filters that each collected air for a 24-h period were obtained from the ranch land, but only one set of four air filters was obtained from the cultivated agricultural land.

At each location all samplers were placed at a height where the collection filter was at 1.2 m (about 4 ft) above the surface. For purposes of this feasibility study, a uniform aerodynamic surface roughness of 15 cm (about 6 in) and a vegetation height of 10 cm (about 4 in) were assumed for both sampling locations.

Meteorological conditions were generally typical for the summer season in Bakersfield, CA dry conditions, temperatures ranging from 21 ° to 35° C (70° to 95° F), and wind most frequently from the north with an average speed of about 4 m/s (9 mi/h).

Harvesting Arthroconidia (Spores) and Demonstration of Spore Killing

To demonstrate that the arthroconidia of *C. immitis* could be killed in order to be handled safely during DNA extraction procedures the following laboratory experiment was conducted. In order to perform this experiment and subsequent calibration studies for the TaqMan™-PCR assay, arthroconidia were needed. These arthroconidia were harvested according to the following process.

Lung tissue from mice previously infected with *Coccidioides immitis* strain Silveira (ATCC 28868) was placed onto agar slants of Difco Mycobiotic Medium (Becton Dickinson, Sparks, MD). This medium contains both cycloheximide and chloramphenicol and is selective for pathogenic fungi (See Difco Laboratories, 1988; and Beneke et al., 1996). Slants were incubated at 37° C until growth appeared. The microorganism was then subcultured onto 2% glucose, 1% yeast extract (2X GYE) agar slants. These slants were incubated at 37° C until growth of the fungus was prevalent and then these slants were placed at room temperature.

The mature arthroconidia were harvested from the 2X GYE slants by gentle agitation of the slant surface with a sterile plastic inoculating loop following the addition of 5 mL of sterile 0.9% sodium-chloride injectable solution (Abbott Laboratories, North Chicago, IL). This cell suspension was then transferred to a second and then a third slant containing mature arthroconidia and in each case the agitation repeated. Finally, this cumulative suspension was transferred to a sterile 50 mL conical polypropylene tube (Falcon, Becton Dickinson). An additional 5 mL of sodium chloride solution was used to wash the slant surface and that was transferred to the second and then the third slants as described above. This wash was added to the previous material in the conical tube. This entire procedure was repeated for an additional three slants. This process produced two tubes, each containing approximately 10 mL of turbid suspension of arthroconidial chains and some mycelial fragments. A single cell suspension was achieved by vortexing the

suspension at high speed after the addition of glass beads (3-mm diameter). The tubes were vortexed for 2-min pulses alternating with incubation on ice to minimize heating. The cell suspension was checked microscopically for chain breakage. This procedure yielded single arthroconidia and small broken mycelial debris. The arthroconidial suspension was then transferred by sterile pipette to a single clean conical tube. The suspension was allowed to rest for 5 min to permit settling of mycelial fragments. The suspension was transferred to a clean tube, being careful not to disturb the settled material. The suspension was then centrifuged at 2000 × g for 20 min. Tubes were enclosed in cups with sealed domes during centrifugation. Following centrifugation, the liquid was removed and the pellet was resuspended in 20 mL of a 0.9% sodium chloride solution. This washing was repeated two times. After the final centrifugation the pellet was resuspended in 5 mL of a 20 mM phosphate buffer at pH 7.0 that also contained 0.003% Tween 20. The resulting cell concentration was estimated using a hemacytometer and found contain about 8.1×10^7 spores/mL. This cell concentration represented the stock solution from which arthroconidial serial dilutions were prepared for both the steam killing and the TaqMan™-PCR assay calibration studies.

From the concentrated stock solution, a dilution was performed to obtain a concentration of 1×10^7 spores/mL and that was then used to perform serial 1:10 dilutions of the spores. All dilutions were prepared in polypropylene tubes using 20 mM phosphate buffer at pH 7.0 and containing 0.003% Tween 20 (phosphate-Tween buffer). The resulting cell concentrations in all dilutions ranged from 1×10^7 spores/mL (lowest dilution) to 1×10^0 spores/mL (highest dilution). The viability of the cells was evaluated by plating 100 mL of the lowest dilution (1×10^7 spores/mL) onto four 2X GYE agar plates. One set of two plates was incubated at 37° C and the other set of two plates was incubated at room temperature. Growth was observed on both sets of plates.

The steam killing procedure was based on the method described by Burt et al. (2000) that was also used by Greene et al. (2000). In this procedure, the lowest dilution of arthroconidia (1×10^7 spores/mL) in the polypropylene conical tubes were placed into metal centrifuge cups with sealed domed lids. This insured that if a tube broke during steaming that the cells would be contained. These cups were then placed into an autoclave chamber and then exposed to steam for 15 min. The autoclave door was left open to prevent pressurization, which might create conditions that would adversely affect the integrity of the DNA and therefore its detection. After 15 min, the steam was turned off and the cups removed and cooled. The tubes were then removed. A 100-µL volume of the lowest dilution (1×10^7 spores/mL) was plated and incubated as previously described. Killing was confirmed by the absence of growth of *C. immitis*.

In addition to proving that arthroconidia could be killed in pure culture, the killing of arthroconidia on air-filters was also examined. To perform this investigation, two "dirty" air filters (used to collect air from a location in Northern California that is not endemic for *C. immitis*) were used to assess the steam killing and the removal of arthroconidia from the membrane. The filters were cut in half and placed into four separate conical tubes (50 mL volume).

Two of these air filters were spotted with 100 µL of viable 1×10^7 spores/mL and were then subjected to the steam treatment to assess the effectiveness of the kill procedure. These two tubes were placed in metal centrifuge cups with domed lids and were steamed at 95° C for 15 min as described previously. After the tubes had cooled, 45 mL of phosphate-Tween buffer was added to each tube and the tubes were then vortexed at high power for 1.5 min. These tubes were then sonicated on power-level 9 in a model 75D aquasonic (VWR, West Chester, PA) for 10 min at room temperature. Following the sonication, the tubes were vortexed for an additional 5 sec and the "dirty" liquid was poured into a clean tube. An additional 45 mL of phosphate-Tween buffer was added to the tube containing the membrane and the vortex/sonication procedure repeated. The liquid was poured into a clean conical tube. The tubes were then centrifuged for 30 min at 2000 × g. The supernatant was removed following centrifugation leaving only enough liquid to resuspend the pellets. The pellets from the two washes were combined, centrifuged, and the remaining supernatant removed. Finally 300 µL of phosphate-Tween buffer was added to give a final volume of 500 µL. A 100-µL volume of the material derived from each half filter was plated onto four Mycobiotic agar plates; two were incubated at 37° C and the other two were incubated at room temperature. No growth was observed on any of these plates, which were held for two weeks.

The remaining two conical tubes containing filters were placed into metal centrifuge cups and were steamed as previously. After the tubes had cooled, 100 µl of the lowest arthroconidia dilution (1×10^7 spores/mL) were spotted onto the steamed membrane. The material and viable arthroconidia were removed from the membrane as described above. This material was spread onto four Mycobiotic agar plates and incubated as described above at either 37° C or at room temperature. Growth was observed on all plates.

Results

The ability to kill *C. immitis* spores so they could be worked with safely for calibrating the TaqMan™-PCR assay and for its application to detection of such spores in environmental air samples was achieved. The data in Table 1 clearly show that *C. immitis* arthroconidia (spores) that were steam treated at 95° C for 15 min, whether as the lowest dilution of harvested spores or as a spike administered to a dirty air filter, do not grow on Mycobiotic agar culture plates, and those spores that are not steam treated do produce growth.

The TaqMan™-PCR assay has been designed to measure pathogens quantitatively in the environment (Haugland et al., 1999). The TaqMan™-PCR assay includes a double-labeled (FAM–TAMRA) fluorogenic probe that is designed to hybridize to a specific target-DNA sequence between two PCR primers, which define the target sequence. The fluorescent-reporter dye FAM is covalently linked to the 5′ end of the probe and is quenched by TAMRA, located at the 3′ end of the probe. As primer extension occurs, the 5′ to 3′ exonuclease activity of Taq polymerase cleaves the probe between the reporter and quencher dyes, but only if the probe is hybridized to its target.

Table 1. Results of plate cultures of *C. immitis* arthroconidia (spores) obtained either directly from the lowest dilution of harvested spores, or recovered from dirty air filters that were spiked with *C. immitis* from the lowest dilution of harvested spores, and that either were or were not subjected to steam treatment at 95° C for 15 min.

	Incubation temperature[a]	
Spore sample tested in plate culture	Room temperature	37° C
Arthroconidia from lowest dilution (1×10^7 spores/mL) and no steam treatment	Growth	Growth
Arthroconidia from lowest dilution (1×10^7 spores/mL) and steam treatment	No growth	No growth
Arthroconidia from lowest dilution (1×10^7 spores/mL) spiked onto a dirty air filter and then recovered after the spiked filter was steam treated	No growth	No growth
Arthroconidia from lowest dilution (1×10^7 spores/mL) spiked onto a steam treated dirty air filter and then recovered (without further steam treatment)	Growth	Growth

[a]Culture plates were held for 2 weeks or until growth appeared, whichever came first.

As cleavage occurs the reporter is no longer quenched and a fluorescence emission signal is emitted, which is a quantifiable expression of the amount of DNA present in a sample (Perkin-Elmer Applied Biosystems, 1996). It is the starting cycle time (Ct) of this amplification signal above a threshold level that indicates the presence of a specified amount of *C. immitis* DNA. No appearance of a fluorescence emission-signal intensity above a threshold level by about 35 amplification cycles (i.e., a starting amplification cycle time of 35 Ct) is considered a negative result.

The specificity of the TaqMan™-PCR assay for identifying *C. immitis* DNA was tested for five *C. immitis* strains, and the ability to distinguish *C. immitis* DNA from the DNA in four strains of *Ucinocarpus* (a closely related species found in the same environment), as well as from a panel of DNA from yeast and *Trichoderma* fungi, and from rabbit, rat, bovine, pig, chicken, human, dog, and mouse. The assay was also checked for specificity against a negative control (water), and the complex background DNA obtained from the microorganisms in air and soil samples from locations in northern California where *C. immitis* is not expected to occur. All of these tests were performed in duplicate. Non-*C. immitis* DNA, including that from the complex

background DNA (and the negative control), was negative in the TaqMan™-PCR assay, while the *C. immitis* strains yielded positive amplification results in the TaqMan™-PCR assay.

The TaqMan™-PCR assay was evaluated for its sensitivity by challenging it with known quantities of steam-killed *C. immitis* spores that were spiked into air-sludge and then recovered (see *Materials and Methods*). In field samples, detection of *C. immitis* DNA is performed in a background of DNA obtained from other microorganisms collected on the air-filter media; in other words, the DNA from all microorganisms are extracted simultaneously. To simulate this condition in evaluating the sensitivity of the TaqMan™-PCR assay, we collected the debris, particles, and microorganisms (i.e., air sludge) deposited on 20 air filters. These filters were produced by air sampling in a location in northern California where *C. immitis* is not expected to occur. The recovered air sludge was then used as the background substrate for inoculation of known amounts of *C. immitis* spores. That *C. immitis* DNA was then co-extracted to establish a calibration curve suitable for its quantification. In Fig. 2 output is shown from a successful TaqMan™-PCR assay showing the cycle time (Ct) at which amplification begins [i.e., where the initial fluorescent signal has a response intensity that is above threshold (+ ΔRn)] for DNA extracted from different known quantities of *C. immitis* spores inoculated onto air sludge. The data in Fig. 2 indicate spore number decreases logrithmically with increasing Ct [i.e., the starting amplification cycle time (Ct)]. The corresponding calibration curve is shown in Fig. 3, and the least-squares exponential fit to the data is defined by Eq. 1,

$$\text{Spore count} = (9 \times 10^{11}) \times e^{(-0.712\,Ct)}, \qquad (1)$$

with $R^2 = 0.9891$.

However, efforts to reproduce the data for the calibration curve shown in Fig. 3 were not successful. It is likely that this difficulty is a consequence of a confounding interference occurring in the TaqMan™ system that is related to conditions occurring in subsequent analyses. Such interference is not obvious or easily determined and therefore will require some further investigation. Yet, it would appear this calibration experiment achieved a sensitivity as low as 1×10^3 spores (see Fig. 2).

Gel electrophoresis using a Low DNA Mass™ Ladder (Invitrogen, Carlsbad, CA) was employed to quantify the mass of DNA extracted from specific numbers of spores. Two extractions of DNA (see Materials and Methods) were performed from a pure culture of *C. immitis* containing an estimated 1.6×10^7 spores (as determined by hemacytometer). Low DNA Mass™ Ladder results were then obtained for these extractions. The Low DNA Mass™ Ladder results were compared to published values by Brody et al. (1989), Prade et al.(1997), and Timberlake, (1978) for spore-to-DNA mass ratios for a close fungal analogue (e.g., *Aspergillus nidulans*) to *C. immitis*. Based on the genomic content of *Aspergillus nidulans,* we would expect to extract 500 ng of DNA from

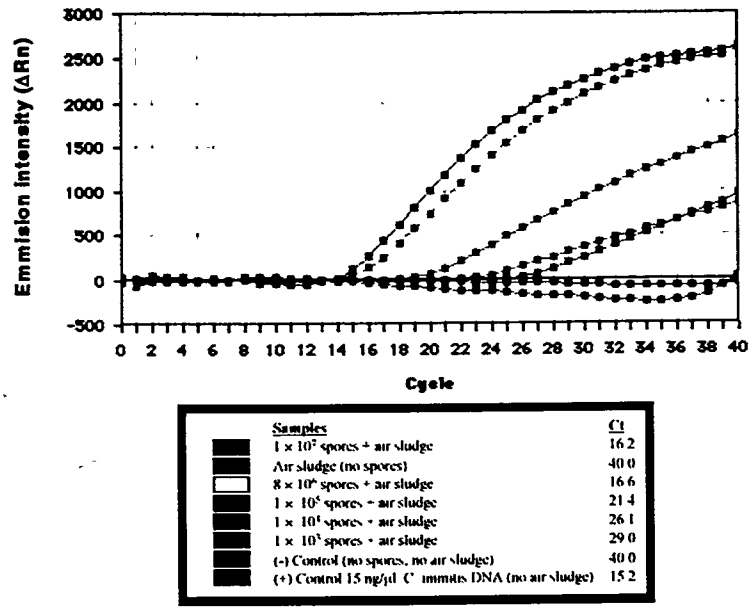

Figure 2. Taqman™-PCR polymerase-chain-reaction (PCR) assay showing amplification of DNA extracted from different known numbers of *Coccidioides immitis* spores after inoculation into and recovery from air-filter concentrate (sludge) and comparison to controls. Earliest cycle time (Ct) where an amplification signal exceeds zero emission intensity corresponds to the unique DNA content of that sample, which is related to the number of spores that are present. A sensitivity as low as 1×10^3 spores was achieved.

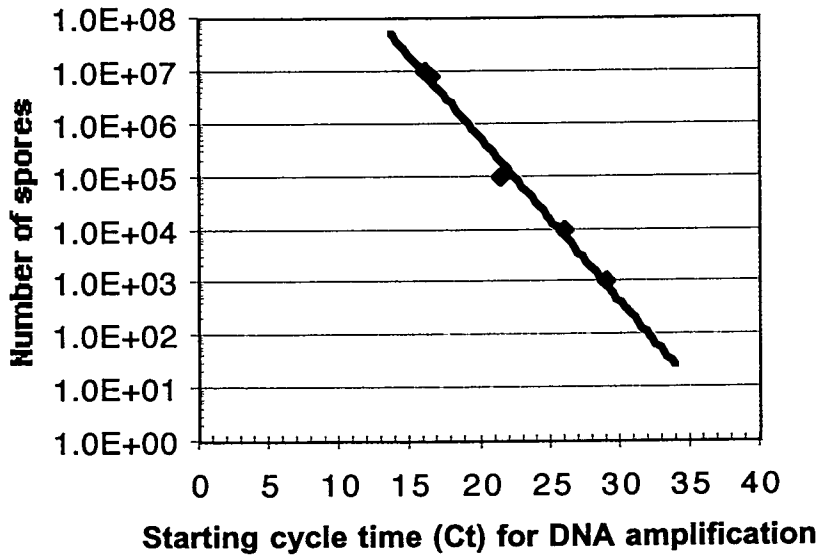

Figure 3. Calibration curve for spores of *C. immitis* in air sludge.

1.6×10^7 *C. immitis* spores; in our extractions we obtained 60 and 140 ng of DNA based on Low DNA Mass™ Ladder results. The efficiency of extraction is the mass obtained from our extractions divided by the theoretical mass of total DNA extraction. The extraction efficiency percentage for our procedure was estimated to be an average of about 20% [i.e., the sum of (60 ng/500 ng) and (140 ng/500 ng) divided 2 and then multiplied by 100]. Fungal spores are notoriously difficult to break open, and this result seems favorable.

In performing air sampling in the field, the upwind area of the ground surface most likely to influence the air collector is of great interest as the soil is the origin for airborne *C. immitis* spores. Schuepp et al. (1990) have derived analytical solutions to predict the upwind area of the ground surface most likely to influence an air sampler. These equations have been applied to high-volume air samplers by Shinn and Gouveia (1992), and are used here for the same purpose, but with general assumptions. For example, a uniform aerodynamic surface roughness of 15 cm (about 6 in) and a vegetation height of 10 cm (about 4 in) were assumed for both sampling locations. Additionally all samplers were placed at a height where the collection filter was at 1.2 m (about 4 ft) above the surface.

The equation used to determine the cumulative normalized contribution to dust flux (*CNF*) for an upwind distance (x_L) is

$$CNF = e^{\frac{B(z-d)}{x_L}}, \qquad (2)$$

where B is related to surface roughness, z is the height of the sampler (1.2 m), and d is the displacement height of the surface (assumed to be 0.07 m). The values of *CNF* can range from just greater than zero to one, and represents the cumulative contribution made to the material on the collector surface from the flux of material an upwind distance (x_L) from the sampler.

For the sampling performed in Bakersfield, CA, in August 2001, the influence of atmospheric stability on the dispersion of the soil flux can be neglected. The value of B we used is a simplification based on that assumption (see Eq. 3).

$$B = \left(\ln Z - 1 + \frac{1}{Z}\right) / k^2 \left(1 - \frac{1}{Z}\right), \text{ where} \qquad (3)$$

$Z=(z-d)/z_0$, k=0.4, and z_0 is the surface roughness (assumed to be 0.15 m for the short grasses and agricultural cultivation at the two different sites, respectively). For the samplers deployed in Bakersfield, CA, in August 2001, Z = 75.3 and B = 21.3.

Inserting the values for *B* and *(z—d)*into Eq. 2, the *CNF* can be calculated for any distance upwind (x_L). Thus, 50% of the material on the collector surface is predicted to come from the flux of material from the ground surface over a distance of 34 m from the collector. Similarly, 90% of the material on the collector surface is calculated to come from the flux of material from the ground surface over a distance of 227 m from the collector.

Furthermore, the distance to the point of maximum contribution to the sample (x_{max}) can be calculated with the following simple expression (from Schuepp et al., 1990) for the collectors deployed in Bakersfield, CA:

$$x_{\max} = \frac{B(z-d)}{2}. \qquad (4)$$

Accordingly, this distance is estimated to be 12 m, and represents the location of the peak for relative-flux-density as measured by the sample. Such a calculation has implications for finding the ground source of the *C. immitis* spores that may be responsible for the spores appearing on an air filter. Such an originating source for *C. immitis* spores has typically been elusive to find in the field, even in endemic areas.

The field samples obtained on the air filters from the two locations in Bakersfield, CA, in August 2001, were processed (see Materials and Methods) and the TaqMan™-PCR assay was employed to determine if *C. immitis* spores were present. Table 2 contains the results of these TaqMan™-PCR assays. For every extraction volume that is obtained from an air filter, triplicate analyses were performed. Amplification cycle times (Ct) that are much less than 35 Ct in *all three* replicates strongly suggest that *C. immitis* is present in that particular air sample. This occurs for the 24-h sample obtained on August 28 from air-sampler No. 4 that was located on the ranch land, and for the 24-h samples obtained on August 29 from air-samplers No. 2 and No. 4 that were located on the ranch land, and from air-sampler No. 7 that was located on the cultivated agricultural land. Although samples obtained on August 29 from air-sampler Nos. 1 and 3, which were located on the ranch land, and from air-samplers No. 5 and 6, which were located on the cultivated agricultural land, may also have contained *C. immitis* spores, the TaqMan™-PCR results did not satisfy the subjective criteria for this study of having to have all three replicates report positive results in order to have a high confidence that *C. immitis* was probably present on the air filter. For comparison, Table 2 also presents the results from positive and negative controls that were also run using the TaqMan™-PCR assay.

According to the data presented in Table 2, the air samples with a high likelihood of containing *C. immitis* spores have average Ct values of 22.1, 20.5, 15.6, and 14.6. According to Eq. 1, obtained from the calibration curve (see Fig. 3), the spore counts corresponding to these average Ct values would equal 1.3×10^5, 4.1×10^5, 1.4×10^7, and 2.8×10^7 spores, respectively. According to application of Eq. 2 and Eq. 3, 50% of these spores would have come from the ground surface up to 34 m from these collectors.

Table 2. Results of TaqMan™-PCR analyses for *C. immitis* on air-filter samples collected from ranch land and cultivated agricultural land in Bakersfield, CA, during August 2001.

Collection Date or control	**Sampling location in Bakersfield, CA (air-sampler identification number)**	**Starting amplification cycle time (Ct, for triplicates using a 5-µL volume of DNA extract)**	**Arithmetic mean Ct (where all three triplicates report detection)**
28 Aug 01	Ranch land (1)	35, 35, 35	Represents no detection
28 Aug 01	Ranch land (2)	35, 35,35	Represents no detection
28 Aug 01	Ranch land (3)	35, 35, 35	Represents no detection
28 Aug 01	Ranch land (4)	19.3, 22.2, 24.7	22.1
29 Aug 01	Ranch land (1)	35, 16.9, 17.2	Not determined
29 Aug 01	Ranch land (2)	21.7, 14.0, 25.8	20.5
29 Aug 01	Ranch land (3)	19.5, 24.7, 35	Not determined
29 Aug 01	Ranch land (4)	18.8, 13.1, 14.8	15.6
29 Aug 01	Cultivated agricultural land (5)	17.0, 16.2, 34.3	Not determined (because Ct = 34.3 considered to represent a no detection)
29 Aug 01	Cultivated agricultural land (6)	35, 14.7, 14.7	Not determined
29 Aug 01	Cultivated agricultural land (7)	13.6, 14.2, 15.9	14.6
29 Aug 01	Cultivated agricultural land (8)	35, 35, 35	Represents no detection
Negative Control: mixture of air sludge from 20 filters without *C. immitis*	Not applicable	35, 35, 35	Represents no detection
Positive Control: mixture of air sludge from 20 filters with 12 ng/µl of *C. immitis* DNA	Not applicable	10.7, 10.9, 11.4	11

Technical Achievements and Conclusion

The technical achievements obtained in this study are significant. Importantly, the results clearly show that *C. immitis* spores can be steam killed successfully in culture and on air-filter membranes, without adversely affecting the DNA, ensuring that the samples can be safely manipulated and analyses by TaqMan™-PCR assay can be performed. They also demonstrate that with a little more research, LLNL should possess the unique capability to reproducibly measure quantitatively in environmental samples, especially from air, the presence of *C. immitis*, which is responsible for coccidioidomycosis and also listed as a select agent. The supporting evidence is that there is a high likelihood that there are *C. immitis* spores present in air-filter samples for which the TaqMan™-PCR assay reports positive results in three replicate samples of DNA extract. This evidence also strongly suggests that the high-volume air-sampler systems, which include the track-etched (1-μm pore size), 8 × 10-in (about 20 × 25-cm), polyester-membrane filter (Osmonics, Westborough, MA) are suitable for use in investigating the ecology of the pathogenic fungus, *C. immitis,* in the environment.

On the basis of the results obtained in this study, it is also interesting to note that the cultivated agricultural field, as well as the relatively undisturbed cattle ranching land, both showed presumptive evidence of the presence of *C. immitis* spores. Although further analyses are needed to improve the fidelity of these results, if *C. immitis* spores are present on the cultivated agricultural land, then this would suggest irrigation and cultivation alone are not necessarily effective in eliminating the source of *C. immitis*, and other interventions may be needed to reduce or eliminate exposure to *C. immitis*.

One way to confirm the presence of viable *C. immitis* spores on the air samples would be to remove the spores and inject them into an animal model (e.g., mice). If the animal model showed signs and symptoms of the disease, and the microorganism could be recultured from the animal tissues, then there would be unequivocal proof that the air-filters contained *C. immitis*. The next step would be to look for *C. immitis* in the soil in the vicinity of the collector and test those samples for the presence of *C. immitis* using the TaqMan™-PCR assay. Once identified in the soil, a study of the relationship between *C. immitis* and ecological, climatic, and geochemical conditions could be started and eventually a greater understanding of why, when, and where this fungus occurs would be achieved.

Now LLNL, and its collaborators at the School of Medicine of the University of California, Davis, are in the favorable position of potentially possessing a unique tool for studying *C. immitis* in the environment. Such a capability is beneficial and can eventually be applied to studying the ecology of other environmentally significant infectious diseases. More importantly, *C. immitis* is unique to California and the southwestern United States, so the LLNL/UC Davis collaboration could even now begin to play a role in helping to find ways to reduce or eliminate the occurrence of *C. immitis* environmentally, and in the process, also contribute information that would benefit national security and the environmental, health, and safety mission of LLNL and the United States Department of Energy. Thus, to achieve this goal, a proposal has been submitted to the National Institute of Allergy and Infectious Disease (NIAID) at the

National Institutes of Health (NIH), in Bethesda, MD. That proposal seeks to perform research that would refine the capabilities developed and pursued in this study and then use them to achieve a greater understanding of the complex relationship between the environment and the occurrence of *C. immitis*. The objective of such research would be to acquire the data that would allow proactive environmental intervention strategies to be developed that would protect public health from exposure to *C. immitis* and reduce or eliminate outbreaks of Valley Fever.

References

Brody, H., and Carbon, J. (1989), Electrophoretic Karyotype of *Aspergillus nidulans*, *Proc. Nat. Acad. Sci. 86,* 6260–6263.

Burt, A., Carter, D.A., Koenig, G.L., White, T.J. and Taylor, J.W. (1995), A Safe Method of Extracting DNA From *Coccidioides immitis*, *Fungal Genet. Newsl. 42*, p. 23; Accessed: January 9, 2000, http://www.seqnet.dl.ac.uk/research/fgsc/fgn42/burt.html (Updated: January 7, 2000).

Beneke, E.S. and Rodgers, A.L. (1996), *Medical Mycology and Human Mycoses,* Star Publishing Company, Belmont, CA, pp. 31, 37–38.

Difco Laboratories (1984), *Difco Manual, 10th Edition,* Difco,Laboratories, Detroit, MI, pp. 586–589.

Greene, D.R., Koenig, G., Fisher, M.C., and Taylor, J.W. (2000) "Soil Isolation and Molecular Identification of *Coccidioides immitis*," *Mycologia 92*, 406–410.

Haugland, R.A., Vesper, S.J., and Wymer, L.J. (1999), Quantitative Measurement of *Stachybotrys chartarum* Conidia Using Real Time Detection of PCR Products With The Taqman™ Fluorogenic Probe System, *Mol. Cell. Probes 13,* 329–340.

Miller, D.N., Bryant, J.E., Madsen, E.L., and Ghiorse, W.C. (1999), Evaluation and Optimization of DNA Extraction and Purification Procedures for Soil and Sediment Samples, *Appl. Environ. Microbiol. 65*, 4715–4724.

Pappagianis, D. (1988), 6—Epidemiology of Coccidioidomycosis, *Curr. Top. Med. Mycol. 2*, 199–238.

Perkin-Elmer Applied Biosystems Division (1996), Chapter 7—Theory of Operation, In *ABI PrismTM 7700: Sequence Detection System—User's Manual,* PE Applied Biosystems, Foster City, CA.

Rose, J.B., Huq, A., Lipp, E.K. (2001), *Health, Climate, and Infectious Disease: A Global Perspective,* American Academy of Microbiology, Washington, DC [Technical Report based on *Am. Acad. Microbiol.* colloquium held October 15 to17, 1999, in Tucson, AZ (http://www.asmusa.org/acasrc/aca1.htm)].

Schuepp, P,H., Leclerc, M.Y., MacPherson, J.I., and Desjardins, R.L. (1990), Footprint Prediction of Scalar Fluxes From Analytical Solutions of the Diffusion Equation, *Boundary-Layer Meteorol. 50,* 355–373, 1990.

Shinn, J.H., and Gouveia, F.J. (1992), *The "Footprint" Area Influencing a High-Volume Air Sampler,* Lawrence Livermore National Laboratory, Livermore, CA, UCRL-ID-112181.

Timberlake, W.E. (1978), Low repetitive DNA Content in *Aspergillus nidulans, Science 202,* 973–975.